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The relevance of the phosphatidylinositolphosphat-binding motif FRRGT of Atg18 and Atg21 for the Cvt pathway and autophagy

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Abstract **Atg18 and Atg21 are homologous *S. cerevisiae* autophagy proteins. Atg18 is essential for biogenesis of Cvt vesicles and autophagosomes, while Atg21 is only essential for Cvt vesicle formation. We found that mutated Atg18-(FTTGT), which lost almost completely its binding to PtdIns3P and PtdIns(3,5)P_2, is non-functional during the Cvt pathway but active during autophagy and pexophagy. Since the Cvt pathway does not depend on PtdIns(3,5)P_2, we conclude that the Cvt pathway requires binding of Atg18 to PtdIns3P. Mutated Atg21-(FTTGT) is inactive during the Cvt pathway but showed only partly reduced binding to PtdIns-phosphates, suggesting further lipid binding domains in Atg21. GFP-Atg18-(FTTGT) and Atg21-(FTTGT)-GFP are released from vacuolar punctae to the cytosol.**

Keywords: Cvt pathway; Proaminopeptidase I; Autophagy; Phosphatidylinositolphosphate; Lipid binding; FRRGT motif

1. Introduction

During starvation autophagy delivers cytosolic material and organelles to the lysosome (vacuole) for degradation [1–3]. The molecular characterization of autophagy started in *Saccharomyces cerevisiae* [4–6], meanwhile 29 ATG (autophagy) genes were identified [7]. Autophagy plays a role during cancer, cardiomyopathy, Huntington's and Parkinson's disease, the removal of intracellular pathogens, ageing, the presentation of antigens via the MHC class II and autophagic death [8–10]. Autophagy starts with the biogenesis of autophagosomes, transport vesicles limited by two membranes. Autophagosomes then fuse with the vacuole and a still membrane-limited autophagic body is released into the vacuolar lumen. Finally the autophagic body is lysed dependent on the lipase-like Atg15 [11] and the cytosolic content is degraded. The preautophagosomal structure (PAS) acts as a donor compartment for the biogenesis of autophagosomes [12,13]. Many Atg proteins colocalize at this site, amongst others: (i) a phosphatidylinositol 3-kinase complex; (ii) Atg5 covalently linked to Atg12; (iii) Atg8 covalently coupled to phosphatidylethanolamine (Atg8-PE); (iv) the serine/threonine kinase Atg1 and (v) the integral membrane protein Atg9. Atg9 cycles between the PAS and mitochondria dependent on the Atg1/Atg13 complex, Atg2 and Atg18 [14,15].

In *S. cerevisiae* the Cvt pathway is a selective variant of autophagy. It is active under nutrient-rich conditions and targets proaminopeptidase I and α-mannosidase to the vacuole. Cvt vesicles are also limited by two membranes, but are smaller than autophagosomes and exclude cytosol. Most Atg proteins function in both the Cvt pathway and autophagy, however Atg proteins specific for one of the pathways were also identified.

The biogenesis of Cvt vesicles and autophagosomes differs from those of classical transport processes. Characterization of Atg18 and Atg21 is promising to gain insight into these mechanisms. Atg18 and Atg21 share sequence homology, but while Atg18 is essential for both autophagy and the Cvt pathway, the function of Atg21 is only essential for the Cvt pathway [16–20]. Atg18 and Atg21 are WD-40 repeat proteins, expected to fold as seven bladed β-propellers. Atg18 locates at punctae of unknown function at the vacuolar membrane and in part at the PAS. The PAS pool of Atg18 is thought to recruit Atg2 to the PAS and to act in cycling of Atg9 between the PAS and mitochondria [15,14]. Further on Atg18 binds to the vacuolar membrane via PtdIns(3,5)P_2. This pool of Atg18 is essential for retrograde trafficking from the vacuole [21]. Atg18 also binds PtdIns3P [21,19]. Replacement of FRRGT (amino acids 284–288) by FTTGT decreased the PtdIns(3,5)P_2 affinity of Atg18 40-fold, abolished its function in retrograde transport and led to its release to the cytosol [21,19]. The FRRGT motif is conserved in Atg21, and Atg21 also binds PtdIns(3,5)P_2 and PtdIns3P [21,19]. Beside the similarities between Atg18 and Atg21 there are also striking functional differences. Atg21 is not involved in retrograde transport from the vacuole, and Atg18 and Atg21 have different functions in the organization of the PAS. Under nutrient-rich conditions, Atg21 is essential for the recruitment of Atg8 and the Atg5–Atg12 conjugate to the PAS, reflecting its essential role in the Cvt pathway [20,19]. Whereas Atg18 is needed for recruitment of Atg2 to the PAS.

Here we analyze the relevance of the FRRGT motifs of Atg18 and Atg21 for the Cvt pathway and autophagy. We found that the reduced PtdIns-phosphate binding of Atg18-(FTTGT) abolished its function during the biogenesis of Cvt vesicles. Most interestingly, its function during autophagy and pexophagy is not blocked. Beside the somewhat controversial findings about Atg1 [22,23] this is the first time that the functions of an Atg protein during the Cvt pathway and autophagy could be separated. We further generated an Atg21-(FTTGT) variant. Our findings suggest that the lipid binding site of Atg18 and Atg21 differ significantly. For binding of PtdIns3P and PtdIns(3,5)P_2 to Atg18 the FRRGT motif is sufficient, Atg21 in contrast strongly requires additional motifs.

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2. Materials and methods

2.1. Strains, media, and growth conditions

Standard media were used [24]. Starvation medium was SD(−N) (1.7% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose). Antibodies were horseradish peroxidase (HRPO)-conjugated goat anti-rabbit (Medac, Hamburg, Germany) and HRPO-conjugated goat anti-mouse (Dianova, Hamburg, Germany); anti-proaminopeptidase I [16]. Fox3 antibodies were from R. Erdmann, Bochum, Germany.

Chemicals: PMSF (Sigma, Deisenhofen, Germany); oligonucleotides (Operon, Germany), other analytical grade chemicals were from Sigma or Merck (Darmstadt, Germany). The ECL detection kit (Amersham, Braunschweig, Germany) and a LAS-3000 imaging system was used for immunoblots.

Strains: WCG4a Matα *ura3 his3-11,15 leu2-3,112* [5]; YMTA WCG4a *pep4*Δ::*HIS3* [5]; YHB1 WCG4a *atg18*Δ::*KAN* [17]; YHB4 WCG4a *atg21*Δ::KAN [16]; BY4741*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* Euroscarf Collection, Frankfurt Germany.

2.2. Plasmids

pRS316-Atg21-HA-EGFP. The Atg21-HA-EGFP plasmid with the native promotor was constructed via homologous recombination using a PCR fragment created from the pUG35 plasmid (J.H. Hegemann, Duesseldorf) with the primers Atg21-HA-EGFP f (cagtgaattgtaatacgactcactatagggcgaattggagGACTCACTATAGGGCGAATTG) and Atg21-HA-EGFP r (cgactacgcttacccctatgatgtgc cggattacgcc ggcGATATCAAGCTTATCGATACCG) containing flanking homologous parts to the plasmid cen-Atg21-HA [20]. The GST-Atg21 and the GST-Atg21-(FTTGT) plasmid were constructed using a PCR fragment from pRS316-Atg21-HA-EGFP and pRS316-Atg21-(FTTGT)-HA-EGFP with the primers Atg21-GST f (TAAT*GGATCC*ATGAAAGTATT ACAAT TCAAT CAAG) and Atg21-GST r (TATTA*CTCGAG*T TATGTAAA TTTATTA TTTTT AGTCAGCAC) containing a *Bam*HI or *Xho*I site. The plasmids resulted from a ligation into the *Bam*HI and *Xho*I sites of pGEX-4T-3 (Amersham). GFP-Atg18 and GFP-Atg18-(FTTGT) under the control of the MET25 promotor were from S. Dove, Birmingham, UK.

2.3. Site directed mutagenesis

Site directed mutagenesis of pRS316-Atg21-HA-EGFP was carried out using the QuikChange®II Site-Directed Mutagenesis Kit (STRATAGENE, La Jolla, CA) following the instruction manual. Mutagenic primer: 5′ Primer: gg tca ctg ttt aaa gaa ttt aca acg ggt acc aga ttg tgc, 3′ Primer: gca caa tct ggt acc cgt tgt aaa ttc ttt aaa cag tga cc.

2.4. Measurement of pexophagy

According to [25], logarithmically growing cells were shifted to synthetic glycerol medium (0.67% yeast nitrogen base without amino acids, 50 mM MES, 50 mM MOPS, 3% glycerol, 0.1% glucose, pH 5.5) for 4 h at 30 °C. Then a 10× yeast extract/peptone solution was added to a final concentration of 1% yeast extract and 2% peptone, and the cells were incubated for 4 h. The cells were then washed and transferred to YTO (0.67% yeast nitrogen base without amino acids, 0.1% Tween 40, 0.1% oleic acid) for 15 h for peroxisome induction. To induce peroxisome degradation cells were shifted to SD(−N). Aliquots were taken at the indicated times and prepared for immunoblot analysis using antibodies against Fox3p.

2.5. Accumulation of autophagic bodies

Cells grown to stationary phase were washed twice with water and shifted to SD(−N) with and without 10 mM phenylmethylsulfonyl fluoride. Photos were taken using Nomarski optics and a Zeiss Axioskope2 microscope.

2.6. Expression of GST-Atg21, GST-Atg21-(FTTGT), GST-Atg18 and GST-Atg18-(FTTGT)

The ATG21 and ATG21-(*FTTGT*) ORFs were cloned into pGEX-4T-3 (Amersham-Pharmacia). GST-Atg18 (GST-SVP1) and GST-Atg18-(*FTTGT*) (GST-SVP1-FTTG) cloned into pGEX-6P-1 were from S. Dove, UK. Plasmids were transformed into *Escherichia coli* BL21 containing the lysozyme expressing plasmid pLysS (M. Rose, Frankfurt, Germany). Cells containing both plasmids were grown in LB+ampicillin (75 μg/ml) + chloramphenicol (25 μg/ml) to an OD_{600} of 0.5–0.8, induced with IPTG (0.1 mM, 2–4 h, 30°C), harvested, snap-frozen and stored at −80 °C. Cells were resuspended in ice-cold PBS containing protease inhibitor-cocktail (Sigma), 10 mM $MgCl_2$, DNAse I (Invitrogen, Karlsruhe, Germany), 1% Triton X-100. Supernatants from lysed cells (15 min, 8000 × *g*, 4 °C) were applied to glutathione–Sepharose 4B beads (30–50 min, 4 °C). Elution followed the manufacturer's recommendations.

2.7. Lipid blots (PIP-Strips)

PIP-strips (Echelon Biosciences, Salt-Lake City, UT) were blocked for 30 min (2% bovine serum albumin, 50 mM Tris–HCl, pH 7.5, 0.5 mM $MgCl_2$), probed with 100 ng/ml GST-fusion protein (50 mM Tris–HCl, pH 7.5, 0.5 mM $MgCl_2$) for 1 h, followed by an antibody against GST (1:5000 in 2% bovine serum albumin, 50 mM Tris–HCl, pH 7.5, 0.5 mM $MgCl_2$) and an antibody conjugated to horseradish peroxidase (1 h).

2.8. Fluorescence microscopy

Cells were visualized with a Zeiss Axioscope2 microscope and an Axiocam digital camera. For nuclear staining 1 ml of the culture was incubated for 15 min at 30 °C with 10 μl of 2 mM Hoechst 33324 while shaking.

3. Results

3.1. Lipid binding of Atg18 is essential for the Cvt pathway, but not for autophagy

So far the relevance of the lipid binding capabilities of Atg18 on its function during the Cvt pathway and autophagy has not been evaluated. Under non-starvation conditions proaminopeptidase I is selectively targeted via the Cvt pathway to the vacuole, where it is proteolytically matured. During starvation for nitrogen autophagy takes over this transport [26]. Analysis of the maturation of proaminopeptidase I in Western blots therefore is a convenient way to monitor the fidelity of the Cvt pathway and autophagy. As shown in Fig. 1A GFP-Atg18, a fusion protein of Atg18 and the "green fluorescent protein" from *Aequoria victoria* complements the proaminopeptidase I maturation defect in non-starved (lane 6) and starved (lane 5) *atg18*Δ cells, indicating its functionality in the Cvt pathway and autophagy. The GFP-Atg18-(FTTGT) mutant protein generated by site directed mutagenesis of the FRRGT motif (amino acids 284–288) to FTTGT has been shown to be defective in binding PtdIns(3,5)P_2 [21]. GFP-Atg18-(FTTGT) is unable to rescue the Cvt pathway defect of non-starved *atg18*Δ cells (Fig. 1A, lane 4), but functional for autophagy (lane 3). As a control *atg18*Δ cells expressing GFP alone were included. Since a partial restoration of the autophagic capacity is sufficient to achieve complete maturation of proaminopeptidase I [20,27], we further analyzed the autophagic rate by incubating the cells in a nitrogen-free medium containing 1% phenylmethylsulfonyl fluoride (PMSF). In the presence of the proteinase B inhibitor PMSF autophagic bodies are not degraded within the vacuole and thus can be visualized in light microscopy. *atg18*Δ cells expressing GFP-Atg18-(FTTGT) showed the accumulation of significant amounts of autophagic bodies in their vacuoles (Fig. 1B). The number of accumulating autophagic bodies is barely quantifiable due to their rapid Brownian movement, however compared to wild-type cells their number seemed to be somewhat reduced.

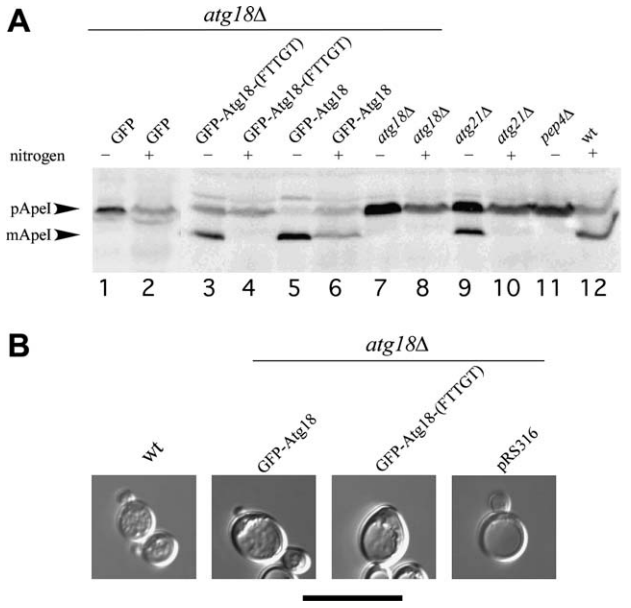

Fig. 1. The FRRGT- motif of Atg18 is essential for the Cvt pathway, but not for autophagy. (A) Crude extracts of *atg18*Δ cells expressing either GFP-Atg18, GFP-Atg18-(FTTGT) or GFP from a plasmid were analyzed by Western blotting with antibodies to proaminopeptidase I. As controls wild-type (wt), *pep4*Δ and *atg18*Δ cells, defective in maturation of proaminopeptidase I and *atg21*Δ cells, only defective in maturation of proaminopeptidase I during Cvt pathway but not during autophagy, are included. Nitrogen (+): log phase cells; Nitrogen (−): cells were starved for 4 h in SD(−N) medium. pApeI: proaminopeptidase I; mApeI: mature aminopeptidase I. (B) GFP-Atg18-(FTTGT) complements the defect of *atg18*Δ cells to accumulate autophagic bodies during starvation with PMSF. The cells were starved for 4 h in SD(−N) medium containing 1 mM of the proteinase B inhibitor phenylmethylsulfonyl fluoride. Photos were taken using Nomarski optics and a Zeiss Axioscope2 microscope equipped with a digital camera. Bar: 10 μm.

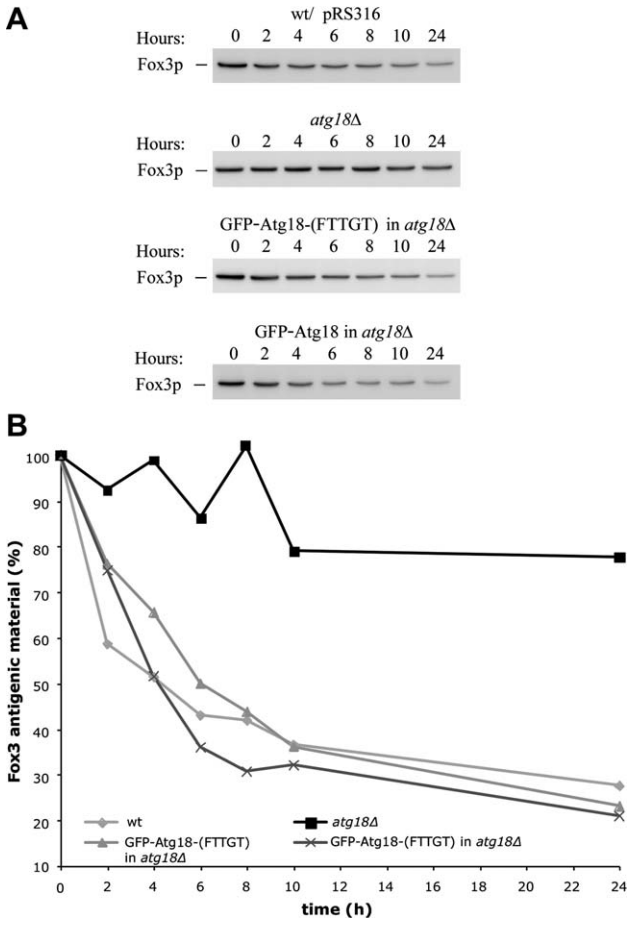

Fig. 2. GFP-Atg18-(FTTGT) is functional during pexophagy. (A) Cells expressing either GFP-Atg18 or GFP-Atg18-(FTTGT) from a plasmid were grown in a medium containing oleic acid to induce proliferation of peroxisomes and then shifted to SD(−N) medium. At the indicated times aliquots are withdrawn and crude extracts of the cells were analyzed by Western blotting with antibodies against the peroxisomal marker protein Fox3. The blots were visualized using a Fuji LAS3000 imaging system. Quantification with the AIDA program package is shown in B. As a control pexophagy deficient *atg18*Δ and wild-type cells were included.

3.2. GFP-Atg18-(FTTGT) is functional during pexophagy

To get a more quantitative assessment of the autophagic functionality of GFP-Atg18-(FTTGT) we next measured pexophagy. Pexophagy is a specialized type of autophagy. Growth of *S. cerevisiae* cells in medium containing oleic acid as carbon source induces the proliferation of peroxisomes. A subsequent shift of the cells to a medium containing glucose but lacking nitrogen results in the breakdown of superfluous peroxisomes by autophagy in a process termed pexophagy [25]. At the indicated times after shift to starvation medium aliquots were taken and the breakdown of the peroxisomes was followed in Western blots with antibodies against the peroxisomal matrix protein Fox3 (3-ketoacyl-CoA thiolase) (Fig. 2A). Direct quantification of the blots using an ECL imager is shown in Fig. 2B. Expression of either GFP-Atg18 or GFP-Atg18-(FTTGT) in *atg18*Δ cells resulted in wild-type like degradation of peroxisomes, suggesting an unaltered activity of GFP-Atg18-(FTTGT) during pexophagy.

3.3. Localization of GFP-Atg18-(FTTGT) in starved cells

In growing cells GFP-Atg18-(FTTGT) has been shown to lose its localization to the vacuolar membrane and to punctae at the vacuole (Fig. 3A) [21]. Our finding that GFP-Atg18-(FTTGT) is functional during starvation induced autophagy and pexophagy opens the possibility that it is still recruited to the PAS under these conditions. We therefore checked its localization in nitrogen-free starvation medium. Beside a weak accumulation in the nucleus, which was also seen in non-starved cells GFP-Atg18-(FTTGT) was cytosolic in starved cells. No obvious accumulation at a dot-like structure reminiscent to the PAS was detectable (Fig. 3B).

3.4. The FRRGT motif of Atg21 is not essential for its binding to phosphatidylinositolphosphates

We were next interested to extend our analysis of the physiological function of the Atg18 FRRGT lipid binding motif during the Cvt pathway and autophagy on Atg21. As outlined in Section 2 we generated a plasmid allowing the expression of a glutathione-S-transferase Atg21 fusion protein (GST-Atg21) in *E. coli*. By site directed mutagenesis we further replaced the FRRGT motif of Atg21 by FTTGT. We purified both versions of the GST-Atg21 fusion proteins with a glutathione affinity column (Fig. 4A) and as a control also included the previously published [21] GST-Atg18 and GST-Atg18-(FTTG) proteins. To check the lipid binding of these proteins we used "dot-blots" [28]. In this approach lipids are spotted onto mem-

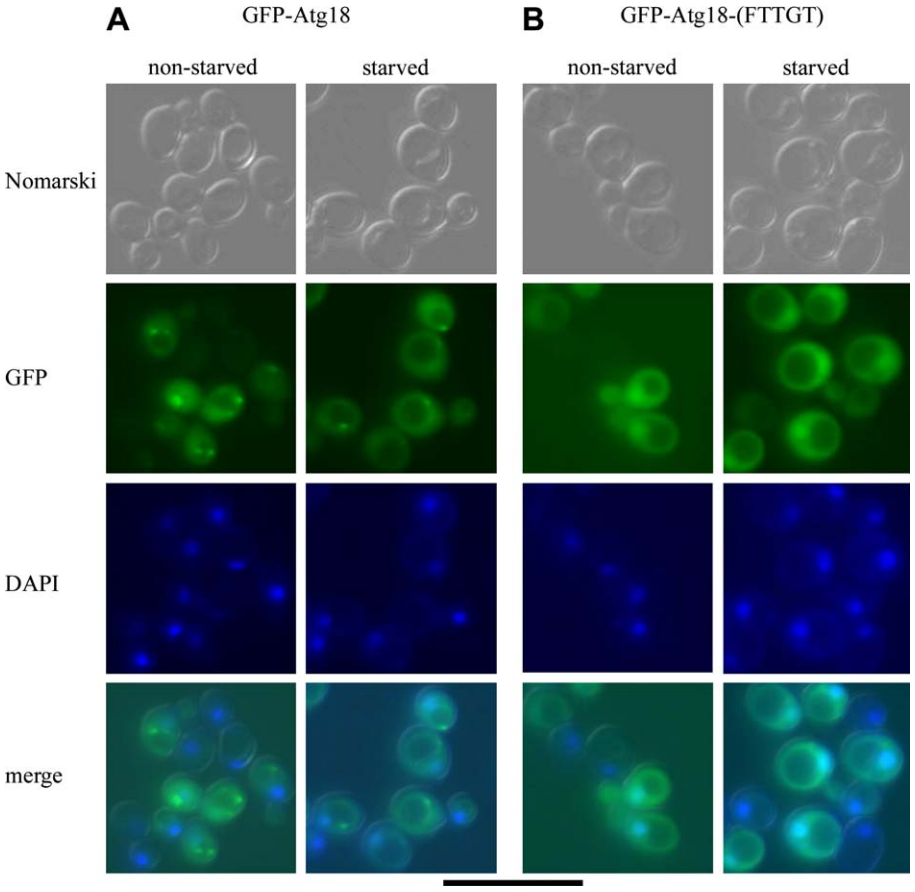

Fig. 3. In starved and non-starved cells GFP-Atg18-(FTTGT) is released to the cytosol. BY4741 cells expressing GFP-Atg18 (A) or GFP-Atg18-(FTTGT) (B) from a plasmid were analyzed by Nomarski and fluorescence microscopy. For starvation cells were incubated for 4 h in SD(−N). Nuclei were stained using Hoechst 33324. Bar: 10 μm.

branes and subsequently incubated with GST-fusion proteins. Lipid-binding proteins can then be identified in a procedure analogous to a Western blot using antibodies to GST. Consistent with published data [21] GST-Atg18 bound to PtdIns3P and to PtdIns(3,5)P_2 (Fig. 4B). In agreement with previous data GST-Atg18-(FTTGT) showed almost no detectable binding to PtdIns(3,5)P_2 (Fig. 4B) [21]. We found here that binding to PtdIns3P is also almost completely blocked. GST-Atg21 bound to PtdIns3P and to a lesser extend to PtdIns(3,5)P_2, PtdIns4P and PtdIns5P (Fig. 4B) [19]. Surprisingly, GST-Atg21-(FTTGT) in contrast to GST-Atg18-(FTTGT) only showed a reduced, but still significant binding to phosphatidylinositolphosphates. Quantification of the ECL signals with an ECL imaging system showed a reduction of the amount of GST-Atg18-(FTTGT) binding to PtdIns(3,5)P_2 to 1.4% (±0.5%), well in agreement with a more than 40 fold decreased affinity measured by plasmon surface resonance by Dove et al. [21]. The binding of GST-Atg18-(FTTGT) to PtdIns3P was reduced to about 3.5% (±3%). It must be noted that the determination of the residual binding of GST-Atg18-(FTTGT) is not very accurate due to the low level of binding. Compared to GST-Atg21 the binding of GST-Atg21-(FTTGT) to PtdIns(3,5)P_2 was reduced to 19% (±4%) and for PtdIns3P to 37% (±4%). This clearly indicates a difference in lipid binding of Atg18 and Atg21. While the FRRGT motif of Atg18 is crucial for its binding of phosphatidylinositolphosphates additional domains within the Atg21 protein are needed.

3.5. The FRRGT motif of Atg21 is essential for the function during the Cvt pathway and its localization

To analyze the importance of the FRRGT motif of Atg21 on its function during the Cvt pathway and its localization we generated plasmids expressing Atg21-GFP and Atg21-(FTTGT)-GFP. As shown in Fig. 5A, lane 6 Atg21-(FTTGT)-GFP is unable to complement the proaminopeptidase I maturation defect of non-starved *atg21*Δ cells. Since Atg21 is not essential for autophagy, starvation for nitrogen rescues the proaminopeptidase I maturation defect (Fig. 5, lane 7). While Atg21-GFP localized to punctate structures near or at the vacuole (Fig. 5B) [20], Atg21-(FTTGT)-GFP was found in the cytosol under both starvation and non-starvation conditions (Fig. 5C). This is reminiscent to the release of GFP-Atg18-(FTTGT) (Fig. 3B), however no obvious accumulation of Atg21-(FTTGT)-GFP in the nucleus was detectable.

4. Discussion

We here analyze the function of the FRRGT motif in Atg18 and Atg21 for the Cvt pathway and autophagy. Our data demonstrate that a Atg18-(FTTGT) mutant protein, whose lipid binding is almost blocked, is still functional during autophagy (Fig. 1B) and pexophagy (Fig. 2), while its function during the Cvt pathway is abolished. Separation of the autophagic function of Atg18 from its function during the Cvt pathway was

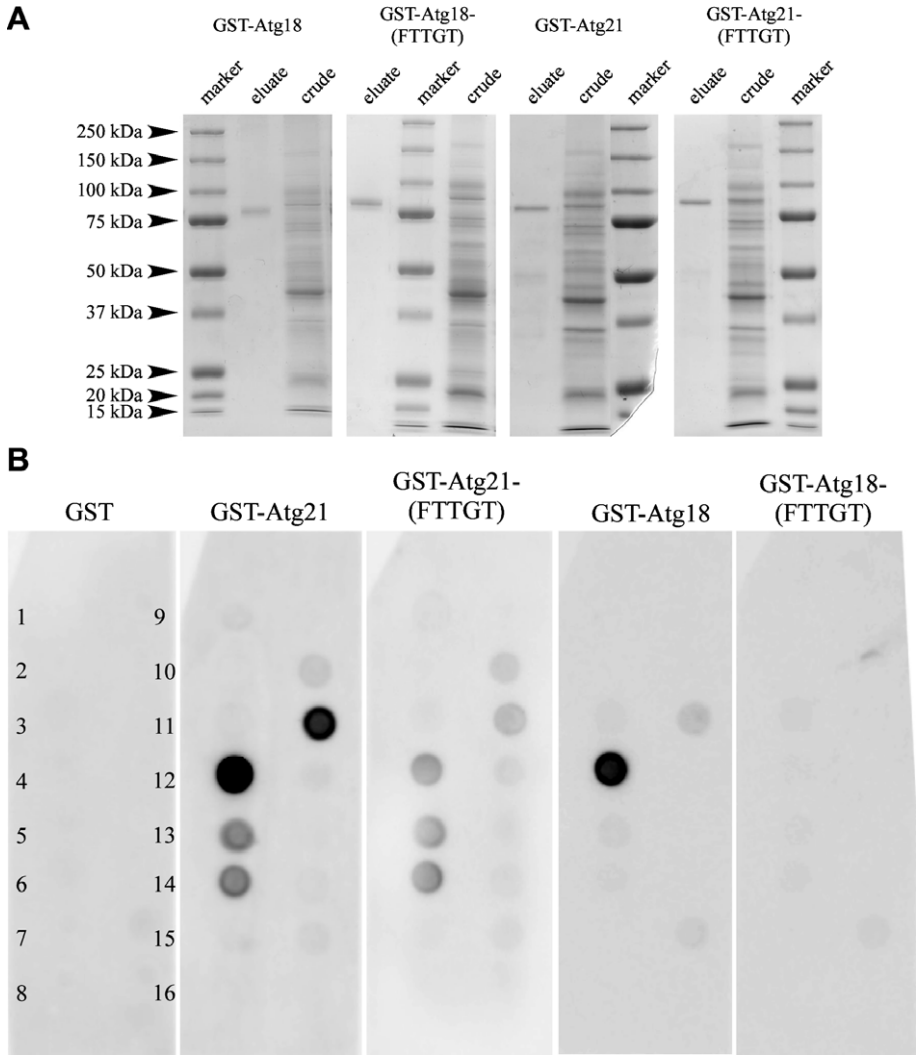

Fig. 4. Site directed mutagenesis of FRRGT to FTTGT in Atg18 and Atg21 affects their lipid binding properties differently. The indicated GST fusion proteins were expressed in *E. coli* and affinity purified using glutathione–Sepharose as detailed. The purity of the isolated proteins were verified by SDS–PAGE and Coomassie staining (A). crude: *E. coli* crude extract; eluate: purified proteins. (B) To determine the lipid binding, the purified GST fusion proteins were incubated with various lipids spotted on membranes. The amount of bound GST fusion proteins was determined in a procedure similar to immunoblotting using antibodies against GST. As a control we included free GST. Lipids spotted onto the membranes: (1) Lysophosphatidic acid; (2) lysophosphocholine; (3) PtdIns; (4) PtdIns3P; (5) PtdIns4P; (6) PtdIns5P; (7) phosphatidylethanolamine; (8) phosphatidylcholine; (9) sphingosine-1-phosphate; (10) PtdIns(3,4)P_2; (11) PtdIns(3,5)P_2; (12) PtdIns(4,5)P_2; (13) PtdIns(3,4,5)P_3; (14) phosphatidic acid; (15) phosphatidylserine; (16) Blank.

rather surprising. To our knowledge the separation of the autophagic and the Cvt function was reported only for the serine/threonine kinase Atg1. Apparently, the Atg1 kinase activity is needed only for the Cvt pathway, but not for autophagy [23]. Another study came to a conflicting result in finding the kinase activity to be essential for both pathways [22]. So far it is expected that during the biogenesis of Cvt vesicles core Atg proteins (which act during both the Cvt pathway and autophagy) are needed together with some Cvt specific helper proteins such as Vac8 and Atg21. The Cvt specific components are no longer essential for autophagosome biogenesis after starvation induction of autophagy, but the core Atg machinery still functions in the same way. Our data are compatible with the idea that at least some of the core Atg proteins might have specific and separable functions during the biogenesis of the individual transport vesicles. This strengthens the view that the Cvt pathway and autophagy are similar, but mechanistically distinct.

Atg18 not only binds to PtdIns3P and PtdIns(3,5)P_2, it further interacts with Atg9 and most likely also with Atg2 [14]. This allows an alternate explanation for the separation of the Atg18 function during autophagy and the Cvt pathway. Probably, the interaction of Atg18 with these proteins is sufficient during autophagy to allow recruitment of Atg18-(FTTGT) to the PAS, but not during the Cvt pathway. The pool of Atg18 at the PAS is rather small [14]. Therefore the release of large amounts of GFP-Atg18-(FTTG) from the vacuolar membrane and punctae near the vacuole into the cytosol might interfere with the detection of a minor protein pool at the PAS in fluorescence microscopy (Fig. 3B). We expect that the function of Atg18 during the Cvt pathway additionally requires its binding to PtdIns3P and not to PtdIns(3,5)P_2 due to two reasons. (i) The yeast PtdIns3P 5-kinase Fab1 is not essential for autophagy [21] and for the Cvt pathway [11]. (ii) An autophagy specific protein complex

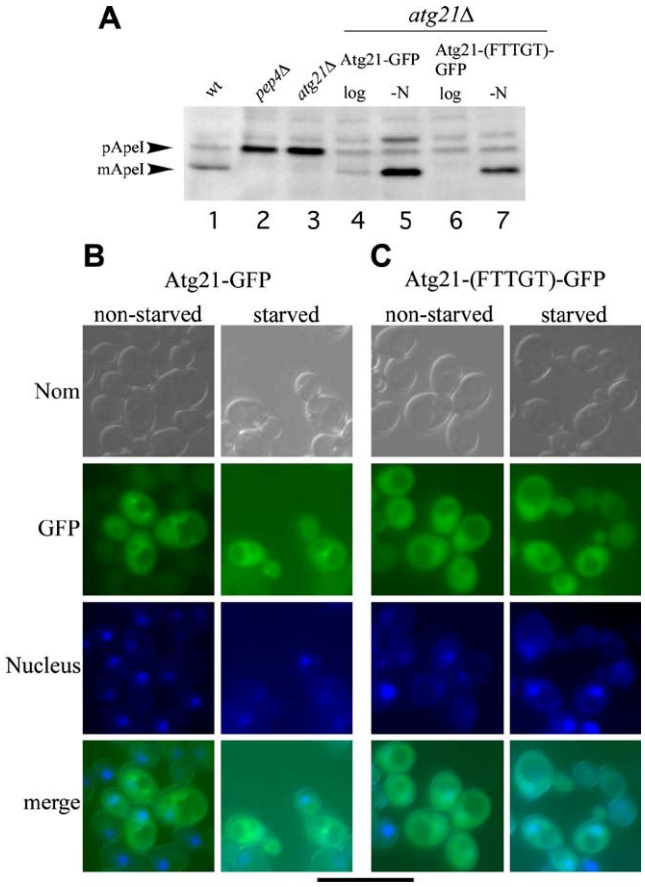

Fig. 5. Atg21-(FTTGT)-GFP is not functional in the Cvt pathway and is released to the cytosol. (A) *atg21*Δ cells expressing either Atg21-GFP or Atg21-(FTTGT)-GFP from a plasmid were processed for Western blotting with antibodies against proaminopeptidase I. Cells were from the logarithmic growth phase (log) or starved for 4 h in SD(−N). As controls wild-type (wt) and *pep4*Δ cells, defective in proaminopeptidase I maturation are included. (B) BY4741 cells expressing Atg21 or Atg21-(FTTGT)-GFP from a plasmid were visualized by Nomarski optics (Nom) and by fluorescence microscopy. Nucleus: nuclear staining with Hoechst 33342. Bar: 10 μm.

containing the PtdIns 3-kinase Vps34 is located at the PAS [29,30].

A recent study identified the lipid binding motif FRRGTY in Atg18 as a potential PKA phosphorylation motif [31]. Therefore it must be taken into consideration that phosphorylation might alter the lipid binding of Atg18 and Atg21. But PKA is not expected to mediate the shift from the Cvt pathway to autophagy, since a lack of PKA activity blocks both the Cvt pathway and autophagy [32].

Compared to Atg18 the FRRGT motif in Atg21 has a drastically reduced relevance, its replacement by FTTGT only reduced the binding of PtdIns3P to ~37% and of PtdIns(3,5)P_2 to ~19% (Fig. 4B). Obviously additional domains of Atg21 are involved in lipid binding. A good candidate would be a region of Atg21, which is missing in Atg18. Atg21 has an additional domain starting at amino acid 40. Atg18 and Atg21 are expected to fold as seven bladed β-propellers [33,21] with the FRRGT motif located within the fifth propeller. The additional domain of Atg21 is proposed to be located between propeller 1 and propeller 2. Interestingly, even the partially reduced lipid binding ability of Atg21-(FTTGT) is sufficient to block its function during the Cvt pathway and to release the protein to the cytosol (Fig. 5). This further demonstrates the strong requirement of lipid binding for the Cvt pathway.

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